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The role of B cells in carriage and clearance of *Mycoplasma pneumoniae* from the respiratory tract of mice

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40-word-or-less summary of article's main point

Mycoplasma pneumoniae (*Mp*) is carried in the upper respiratory tract (URT) of mice after symptomatic infection. B cells and *Mp*-specific antibodies are crucial for *Mp* clearance in the lungs of mice, but are limited in clearing *Mp* from the URT.

Abstract

Carriage of *Mycoplasma pneumoniae* (*Mp*) in the nasopharynx is considered a prerequisite for pulmonary infection. Interestingly, *Mp* carriage is also detected after infection. While B cells are known to be involved in pulmonary *Mp* clearance, their role in *Mp* carriage is unknown. We here show in a mouse model that *Mp* persists in the nose after pulmonary infection, similar to humans. Infection of mice enhanced *Mp*-specific immunoglobulin (Ig) M and IgG levels in serum and bronchoalveolar lavage fluid. However, nasal washes only contained elevated *Mp*-specific IgA. These differences in Ig compartmentalization correlated with differences in *Mp*-specific B cell responses between nose- and lung-draining lymphoid tissues. Moreover, transferred *Mp*-specific serum Igs had no effect on nasal carriage in B cell-deficient μ MT mice, while this enabled μ MT mice to clear pulmonary *Mp* infection. This is

the first evidence that humoral immunity is limited in clearing *Mp* from the upper respiratory tract.

Keywords

Mycoplasma pneumoniae; pneumonia; B cells; antibodies; carriage; mouse model; immunization; vaccine

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Introduction

Mycoplasma pneumoniae (*Mp*) is a frequent cause of childhood community-acquired pneumonia (CAP) worldwide [1], and currently is the most commonly detected bacterial cause of CAP in hospitalized children in the U.S. [2]. We and others observed that besides causing upper and lower respiratory tract (URT and LRT) infection, *Mp* is also carried in the URT of asymptomatic children [3-7]. Carriage is considered a prerequisite for infection [3, 8-10]. Interestingly, carriage also occurs following symptomatic infection [11]. Several studies in humans demonstrated the presence of *Mp* in the nasopharynx up to eight months after respiratory tract infection [3-7].

B cells are of crucial importance for the host defense against multiple pathogens, as B cell activation can result in the production of specific immunoglobulins (Igs) that neutralize the pathogen [12]. Following an initial *Mp* respiratory tract infection *Mp*-specific IgM is detectable in serum within one week, and increased amounts of *Mp*-specific IgG can be found from two weeks post infection (p.i.) [13, 14]. *Mp*-specific IgA is produced early after infection and its levels both peak and decrease earlier than IgM or IgG [15]. Indeed, B cells are known to be involved in pulmonary *Mp* clearance [16-20]. Furthermore, patients with hypogammaglobulinemia are at greater risk for *Mp* pulmonary disease and/or extrapulmonary manifestations [21-25]. It is likely that carriage of *Mp* by these patients make them susceptible to the development of symptomatic *Mp* infection.

In contrast to the known important role of B cells in clearance of a pulmonary infection, limited knowledge exists on the role of B cells in the control of *Mp* carriage in the URT. We therefore set out to determine whether humoral immunity contributes to clearance of *Mp* carriage from the URT. Since this is difficult to study in human subjects, we further developed a C57BL/6 mouse model to study *Mp* (strain M129) infections and the role of humoral immunity on control of these infections.

Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories and used at 8–12 weeks of age. μ MT transgenic mice (C57BL/6 \times FVB \times 129) [26] were bred and housed in the animal facilities of the Erasmus MC under specific pathogen-free conditions. All experiments were approved by the Animal Experiments Committee of the Erasmus MC, Rotterdam, The Netherlands.

Bacteria

Mp reference strain M129 (subtype 1, ATCC 29342) was cultured at 37°C/5% CO₂ in SP4 medium (pH 7.8–8.0) for 72 hours and concentrated in fresh SP4 medium to $1 \times 10^{9-10}$ colony-forming units (CFU)/ml (Supplementary Data). To track *Mp*, bacteria were labeled with 10 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Molecular Probes).

Mp infection and carriage mouse model

Mice were inoculated intranasally with 1×10^9 CFU of M129 diluted in 50 μ l SP4 medium. Control mice were inoculated with 50 μ l SP4 medium. On indicated days after *Mp* inoculation, body weight was measured and mice were sacrificed. Lung washes and nasal fluid samples (washes and brushes) were collected. Cells from lungs, lymph nodes, and nasal passages (mucosa and nasal-associated lymphoid tissue [NALT] [27]) were isolated. Detailed methods for sample procedures are included in Supplementary Data.

Serum transfer

Sera were collected and pooled from wild-type (WT) and μ MT mice on day 14 p.i., diluted with PBS (1:1.3), and 200 μ l was injected intravenously into each WT and μ MT recipient mice at day 15, 16, and 22 p.i. One week after the last serum transfer, recipient mice were sacrificed. Sera were devoid of *Mp* as confirmed by a negative PCR for *Mp* [28].

***Mp* quantification**

The presence of *Mp* was detected either by PCR [28] or culture of bronchoalveolar lavage fluids (BALF) and nasal fluid samples (100 μ l undiluted and 10-fold diluted) on SP4 agar plates at 37°C. Suspensions of spleen, liver, and immersed cotton swabs of articular surfaces were processed in a similar fashion.

***Mp* phagocytosis and killing**

Mp M129 was incubated with sera from infected WT mice. As control, sera from uninfected mice were used. Murine RAW 264.7 macrophages were incubated with the respective bacteria for 20 min, washed, and at time point 0 and 180 min, cells were lysed to determine bacterial load (CFU/ 10^5 RAW cells) by culture on SP4 agar plates.

Histopathology scoring

Histopathology scoring (HPS) of lung sections was performed without knowledge of the treatment or time after infection of mice. The modified HPS system [29] assigns values from 0 to 10 by grading immune cell infiltrates in alveoli and bronchi for distribution, severity of inflammation, presence of necrosis, and presence of perivascular, peribronchial, and peribronchiolar cuffing (Supplementary Data).

Quantification of antibodies

Total antibody concentrations ($\mu\text{g/ml}$) and *Mp*-specific antibodies (arbitrary units [AU]/ml) were quantified by ELISA as described in Supplementary Data.

IgA ASC ELISpot

The frequency of *Mp*-specific and total IgA-producing antibody secreting cells (ASCs) in MLN, CLN, and nasal passages was measured by enzyme-linked immunospot (ELISpot) assay using a commercially available kit (Mabtech) as described in Supplementary Data.

Flow cytometry

Aliquots of single-cell suspensions were incubated with appropriate dilutions of antibodies for 30 min on ice. Cells were subsequently analyzed using a FACS Canto II (BD Biosciences) and FlowJo 10 software (Tree Star). Monoclonal antibodies and the gating strategy are described in Supplementary Figure S1.

Cytokine analysis

Cytokines were measured in BALF and nasal lavage fluid by a multiplex fluorescent-bead-based immunoassay (Procarta multiplex, eBioscience).

Statistical analysis

R software environment (version 3.4.0) and GraphPad Prism (version 5.01) were used for statistical analysis. The Welch's *t*-test, Mann-Whitney *U* test, Kruskal-Wallis test with post hoc Dunn's multiple comparisons test of selected pairs, Fisher exact test or χ^2 test were used to determine statistical significance. Family-wise error rates were controlled using the Holm-

Bonferroni Method. Association between paired samples was tested using the Pearson's product moment correlation coefficient. Statistical significance was defined as $p < 0.05$.

Results

Mp persists in the nose after pulmonary infection

To unravel the role of B cells in *Mp* pulmonary infection and nasal carriage, we further developed a mouse model for *Mp* infection using the *Mp* reference strain M129 (subtype 1) in C57BL/6 mice [30-32]. Intranasal inoculation of anaesthetized C57BL/6 mice with 1×10^9 CFU of *Mp* M129 resulted in a mean relative body weight loss of 4% within three days (Figure 1A). After two weeks, infected mice returned to their initial weight and from then on were comparable to control mice, which did not lose weight at any time point. BALF cultures of infected mice, but not control mice, were positive for *Mp* with the highest median bacterial load of 3×10^4 CFU/ml on day three p.i. (Figure 1B). Bacterial loads in BALF gradually declined within two weeks, and were undetectable at four weeks after infection. In contrast to the lung, *Mp* persisted in the nose. During the first two weeks of infection, median bacterial loads were $5\text{--}9 \times 10^4$ CFU/ml, and at day 42 p.i., *Mp* was still detected in the nasal lavage fluid of 5 out of 6 mice, with a median bacterial load of 6×10^2 CFU/ml (Figure 1C). The only mouse in which *Mp* was not detected in the nasal lavage fluid at day 42 p.i. did show a positive culture for *Mp* when nasal brush samples were analyzed (data not shown).

These findings were corroborated using CFSE-labeled *Mp* M129: CFSE-labeled bacteria were detected on the bronchial epithelium and in alveoli forming islets in the lungs at day one p.i. (Figure 1D). The loss of fluorescence from the lungs of infected mice at day three p.i. likely resulted from bacterial replication, as high bacterial loads were detected in the lungs at this same time point (Figure 1B) and CFSE signals are reduced two-fold at each bacterial division [33]. This was confirmed when sections were incubated with an anti-*Mp* antibody: *Mp* was detected in the lungs both at day one and day three p.i. (data not shown).

By contrast, CFSE-labeled *Mp* was detected on the nasal respiratory epithelium at both time points analyzed, without an apparent loss of fluorescence intensity between these time points (Figure 1E). In contrast to previous studies showing invasion and intracellular survival of *Mp* in vitro [34, 35], we did not observe CFSE-labeled *Mp* within respiratory epithelial cells (Supplementary Figure S2).

Specific IgG antibodies in serum and BALF coincide with clearance of *Mp* from the lungs

To define the course of pneumonia following infection with *Mp* and identify immune mechanisms that contribute to pulmonary clearance, we examined pulmonary inflammation by HPS as well as immune cells and antibodies in BALF at the indicated time points after inoculation of mice with *Mp*. Pulmonary inflammation, demonstrated by both inflammatory infiltrates and necrosis in HPS (Figure 2A) and increased BALF leukocytes (Supplementary Figure S3A), was most severe on day three p.i., and resolved at day 28 p.i. At day three p.i., the infected lungs showed pulmonary consolidation at gross pathology and histopathology showed thickened alveolar septa characterized by infiltration of inflammatory infiltrates and presence of perivascular and peribronchiolar cuffing (Figure 2B). Using flow cytometry, we detected a major influx of granulocytes, monocytes, and dendritic cells (DCs) into BALF at day three p.i. (Supplementary Figure S3B), which corroborated the inflammation seen histologically at that time point (Figure 2). Additionally, increased numbers of CD4⁺ and CD8⁺ T cells, NK cells, and B cells were detected in infected lungs, albeit at much lower frequencies than observed for the innate immune cells (Supplementary Figure S3C).

Since B cells only marginally infiltrated the lungs, we evaluated the systemic and local humoral immune response against *Mp*. Both *Mp*-specific IgM and IgG were detected in serum from day seven p.i. (Figure 3A). While specific IgM levels declined after day 28 p.i., specific IgG persisted at high levels. Further analysis revealed that both IgG1 and IgG2a subclasses were present in the *Mp*-specific IgG pool (Figure 3B), which indicates neutralizing

and complement-dependent killing of *Mp*. Indeed, we found that the serum of *Mp*-infected WT mice had the capacity to opsonize *Mp* resulting in enhanced internalization and killing of the bacteria by macrophages in vitro (Figure 3C).

Notably, *Mp*-specific IgG levels in the nasal lavage fluid were far below those detected in BALF on day 42 (Figure 4A). The opposite was seen for specific IgA: the levels were highly increased in nasal lavage fluid but not in BALF. Total IgG and IgA levels were similar in BALF and nasal lavage fluid (Supplementary Figure S4). ELISpot analysis revealed that *Mp*-specific IgA ASCs were present in CLN and nasal passages seven days p.i., with highest numbers in CLN (Figure 4B). In contrast and in line with the data obtained by ELISA, *Mp*-specific IgA ASCs were not present in MLN.

This prompted us to investigate the activation and expansion of B cells in the cervical and mediastinal lymph nodes (CLN and MLN) that drain the URT and LRT, respectively. B cell expansion and activation was observed in both MLNs and CLNs, but not in peripheral (inguinal) lymph nodes (PLNs), which indicates that *Mp* infection induced a B cell response only locally and not systemically (Figure 4C and 4D). Interestingly, the expression levels of CD86 were higher on B cells in MLN than on those in CLN (Figure 4E). Notably, increased numbers of CD86⁺ B cells were still found in CLN, but not in MLN, on day 28 p.i., albeit not significant from controls. Together, these findings demonstrate a distinct difference in the B cell response to *Mp* between LRT and URT lymphoid tissues.

B cells are essential for pulmonary clearance of *Mp* and affect *Mp* carriage

To further evaluate the contribution of B cells to protective immunity against *Mp*, we assessed whether bacterial replication in the URT and LRT is altered in μ MT mice. Similar to WT mice, an initial body weight loss at day three p.i. was measured in μ MT mice. Yet, while infected WT mice were back at their initial weight within two weeks and from then on continued to gain weight, the weight of μ MT mice remained lower than that of WT mice at all

time points analyzed (Figure 5A). Interestingly, while *Mp* was no longer detectable in BALF of WT mice at day 28 p.i., bacteria were still detectable at day 42 in μ MT mice, albeit at relatively low levels (Figure 5B). In the absence of antibodies mycoplasma is suggested to be more prone to disseminate extrapulmonary [16-18], however, spleen, liver, and articular surfaces of μ MT and WT mice on day 14 and 28 p.i. were negative for *Mp* by culture and PCR (data not shown).

In line with the long-term presence of *Mp* in the lungs of μ MT mice, both the HPS of alveoli (Figure 5D) and the lung leukocyte number (Figure 5E) of μ MT mice was higher than those of WT mice on day three p.i. Moreover, in comparison with WT mice, the lungs of μ MT mice showed even increased numbers of leukocytes on day 42 p.i. (Figure 5E and 5F).

Interestingly, also significantly reduced control of bacterial replication was noted in the URT of μ MT mice (Figure 5C). *Mp* was detected in nasal lavage fluid of all μ MT mice and the median bacterial loads remained at similar levels from day 14 to 42 p.i. (2×10^4 CFU/ml), while median bacterial loads in WT mice decreased from 4×10^4 to 6×10^2 CFU/ml during this time period. In the absence of B cells, significantly higher levels of the proinflammatory mediators interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 were detected in nasal lavage fluid of μ MT mice at day 3 p.i. (Figure 5G).

Serum transfer from WT infected mice to infected μ MT mice only controls bacterial replication in the lungs

To specifically examine the contribution of the IgG and IgM antibodies in serum to clearance of bacteria from the nose, we passively immunized μ MT mice with serum from infected WT mice. Therefore, donor and recipient mice were both intranasally inoculated with 1×10^9 CFU of *Mp* M129. Two weeks later, when recipient WT and μ MT mice still had similar median levels of bacterial loads in the lungs (Figure 5B), donor serum was transferred (Figure 6A). As shown in Figure 6B, μ MT mice were only able to control *Mp* replication in the lungs upon

transfer of serum from infected WT mice, as indicated by the similar bacterial load in the lungs of μ MT and WT mice at day 28 p.i. Transfer of μ MT serum, however, did not reduce *Mp* loads in the lungs of infected μ MT recipient mice; pulmonary *Mp* loads in these μ MT recipients were similar to those of untreated controls at day 28 p.i. (Figure 6B). Moreover, these findings were corroborated by the detection of *Mp*-specific IgG in the BALF of four out of six μ MT recipient mice two weeks after WT serum transfer (Figure 6D). *Mp* persisted in the lungs of the two μ MT recipient mice, whose BALF did not contain *Mp*-specific IgG after serum transfer (Figure 6E).

By contrast, the bacterial loads in the nose were comparable for all recipient μ MT mice (with a median of 2×10^4 CFU/ml), irrespective of the specific source of the serum (Figure 6C). The median concentration of *Mp*-specific and total IgG in nasal lavage fluid of infected μ MT mice after serum transfer was 5 AU/ml and 0.005 μ g/ml, respectively, at day 28 p.i. Notably, there was no significant difference between *Mp*-specific IgG levels in BALF and nasal lavage fluid of μ MT mice receiving WT serum (median 6 vs. 5 AU/ml). In addition to nasal lavage fluid and BALF, *Mp*-specific and total IgG antibodies were also detected in serum of μ MT mice after WT serum transfer (Supplementary Figure S5).

Discussion

Here, we show *Mp* persistence after infection in a further developed mouse model for *Mp* infection [30-32]. B cells in lung-draining MLNs become activated and expand early after *Mp* infection, resulting in elevated levels of *Mp*-specific IgM and IgG antibodies in serum and BALF. This B cell activation in MLNs may be key to clear the infection as shown by our observations in μ MT mice, in which *Mp* infection leads to chronic pulmonary disease, characterized by failure to thrive, more severe pneumonia, and long-lasting innate and adaptive compensatory immune cell infiltrates, due to bacterial persistence in the lungs. Conversely, μ MT mice do clear *Mp* infections in the lungs when passively immunized with

serum from infected WT mice. Additionally, we showed that the induced antibodies enhance phagocytosis and killing by macrophages in vitro, which suggests that they can contribute to resolve *Mp* infections in vivo.

In contrast to the lungs, B cells in the nose-draining CLN did not become activated by the presence of *Mp*. As the draining lymph nodes are the major sites of B cell induction following respiratory tract infection [36], the observed differences in B cell expansion and activation between the nose-draining CLN and lung-draining MLN suggest a different control of bacterial replication in the URT compared to that in the LRT. Indeed, *Mp* persisted in the nose of WT mice while the bacterium was cleared from the lungs during the course of infection. Importantly, these data mimic the findings in humans, where *Mp* is carried in the URT of children up to eight months after symptomatic infection [3-7]. The observed difference in B cell responses between LRT and URT lymphoid tissues is in line with the low levels of *Mp*-specific IgG and IgM in the nasal lavage fluid. It is unlikely that *Mp*-specific IgG does not reach the URT, as total IgG levels were similar in nasal lavage fluid and BALF, and we observed comparable bacterial loads in the URT of WT mice as in that of Bruton tyrosine kinase (Btk)-deficient mice, which have reduced peripheral B cell numbers (data not shown). In contrast to *Mp*-specific IgG, *Mp*-specific IgA was more abundant in nasal lavage fluid than in BALF of *Mp*-infected mice. While this has not been shown before for bacterial infections, similar observations were made in influenza virus-infected mice [37]. The vast majority of ASCs in the URT (NALT) of such mice were found to produce IgA, whereas B cell responses in the LRT (lungs) mainly generated IgG and IgM [38]. In fact, we also detected *Mp*-specific IgA ASCs in CLN and nasal passage, but not in MLN, which is in line with observations from Hodge and Simecka [39], showing mycoplasma-specific IgA ASCs in nasal passages but not in the lungs from mice following nasal immunization. Furthermore, these data also corroborate our findings on the presence of IgA in nasal washes but not in BALF. Detection of *Mp*-specific IgA-producing ASCs in the nasal passage, albeit at lower numbers than in CLN, may indicate that some of the activated B cells have migrated into the nasal passage.

The locally induced immune responses upon *Mp* carriage in the URT and infection of the LRT are distinct. We show that *Mp*-specific IgG controls *Mp* in the lungs and does not impact on carriage in the URT. By contrast, *Mp*-specific IgA and IgA ASCs in the URT affect *Mp* carriage, but do not mediate complete resolution of the bacteria. Using μ MT mice and serum transfer studies we further assessed the relative roles of immunity in the URT and LRT of *Mp* infected mice. Transfer of WT immune serum, containing *Mp*-specific IgG, controlled *Mp* pulmonary infection in lungs of μ MT mice but did not lead to a reduction of *Mp* loads in the nose. Together, these observations demonstrate the importance of local immunity. Yet, why IgA cannot clear bacterial carriage in the URT is unknown [40]. *Mp* is not known to possess IgA protease activity [41]. Also, increasing local specific IgA responses by immunization did not lead to a corresponding reduction of mycoplasma bacterial loads in the nose [39], which suggests that enhancing IgA responses alone does not control carriage in the URT.

Our observation that *Mp* infections are more severe – and chronic – in μ MT mice may resemble the findings in patients that suffer from B cell deficiencies [42], such as common variable immunodeficiency, X-linked agammaglobulinemia or hypogammaglobulinemia, who have been reported to be at increased risk for *Mp* pulmonary disease and/or extrapulmonary manifestations, e.g., arthritis [21-25]. It can be speculated that if these patients experience *Mp* infections, they may benefit from intravenous immunoglobulin (IVIg) treatment, as our data demonstrate that administration of serum containing *Mp*-specific IgG protected μ MT mice from pulmonary *Mp* infections. In fact, high amounts of *Mp*-specific IgG can be detected in commercial human IVIg preparations [43].

While the *Mp* mouse model provides a useful tool to investigate the immunological processes involved in controlling infection of the lungs and carriage in the nose, the use of the human pathogen *Mp* instead of *M. pulmonis* [44] can be a limitation of our study considering the genomic differences between these pathogens [45].

Together, we show for the first time that, like in humans, *Mp* is carried in the URT of mice after symptomatic infection. In addition, we demonstrate that B cells and *Mp*-specific antibodies are crucial for *Mp* clearance in the lungs of mice, but are limited in clearing *Mp* from the URT. These novel aspects of *Mp*-host interactions will aid the understanding of *Mp* pathogenesis and promote the development of *Mp*-targeting vaccines to prevent the progress of *Mp* pulmonary disease in individuals that carry *Mp*.

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Conflict of interest

None.

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Figures

Figure 1. *Mp* persists in the nose after pulmonary infection of C57BL/6 WT mice.

C57BL/6 WT mice were infected intranasally with 1×10^9 CFU *Mp* M129 (black circles) or mock-infected with SP4 medium (white circles). (A) Body weight changes were monitored at indicated time points p.i. and expressed as percentage of body weight at day 0; the mean \pm standard deviation (SD) of each group ($n=6-16$ mice/time point) is shown. $*p<0.05$, $***p<0.001$ (Welch's *t*-test with Holm-Bonferroni method correction). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid; values are expressed as median with interquartile range (IQR). (D–E) Immunofluorescent images of lung and nasal sections of C57BL/6 WT mice ($n=3$ mice/time point) at indicated time points after intranasal inoculation with 1×10^9 CFU CFSE-labeled *Mp* M129 (image: 200 \times magnification; inset: 1000 \times magnification).

Figure 2. *Mp* pneumonia is characterized by alveolar and bronchiolar immune cell infiltrates at day three p.i.

C57BL/6 WT mice were *Mp*-infected (black bars) or mock-infected (white bars). (A) Histopathology score for mice at indicated time points p.i.; bars represent the median \pm IQR of each group ($n=6$ mice/time point). $*p<0.05$ (Kruskal-Wallis test with post hoc Dunn's multiple comparisons test). (B) Representative lung paraffin sections from *Mp*-infected and control mice on day 3 p.i., stained with hematoxylin and eosin (H&E) (image: 50 \times magnification; inset: 200 \times magnification).

Figure 3. *Mp*-specific serum antibodies have neutralizing capacities. (A–B) *Mp*-specific

antibody titers in serum of *Mp*-infected (black circles) and control treated (white circles) C57BL/6 WT mice ($n=6-16$ mice/group/time point): (A) *Mp*-specific IgM, IgG, and IgA at indicated time points; (B) *Mp*-specific IgG subclasses IgG1 and IgG2a at day 42 p.i. Dots represent individual mice and the horizontal line in each graph the median. (C) *Mp* was incubated with sera from infected (dark gray box) or uninfected (light gray box) C57BL/6 mice

or with medium (white box), and subsequently co-cultured with RAW cells for 20 min. After washing, phagocytosis and killing of *Mp* was determined based on the number of CFU/10⁵ RAW cells recovered. The box represents the lower and upper quartiles and the median is shown as a line across the box. Whiskers extend to the maximum or minimum values within 1.5 times the IQR above and below the 3rd and 1st quartile, respectively. * $p < 0.05$, ** $p < 0.01$ (A: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test; B: Mann-Whitney *U* test).

Figure 4. Differential B cell activation in upper versus lower respiratory tract. (A) Analysis of *Mp*-specific IgG and IgA antibody titers in BALF and nasal lavage fluid at 42 days p.i. (IgG) and 28 days p.i. (IgA) of *Mp*- and mock-infected C57BL/6 mice ($n=4-6$ mice/group/time point). (B) Analysis of *Mp*-specific and total IgA antibody-secreting cells (ASCs) in MLNs, CLNs, and nasal passages at 7 days p.i. of *Mp*-infected C57BL/6 mice ($n=3-6$ mice). (C) Number of CD19⁺MHCII⁺ B cells in MLNs, CLNs, and inguinal PLNs. (D–E) CD86⁺ B cell numbers (D) and mean fluorescence intensity (MFI) (E) as ratio to medium controls in MLNs and CLNs. Bars and horizontal lines indicate the median and dots individual mice. * $p < 0.05$, ** $p < 0.01$ (A: Mann-Whitney *U* test; B–E: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test).

Figure 5. μ MT mice show limited pulmonary clearance of *Mp* and higher nasal carriage rates. C57BL/6 WT mice (black circles) and μ MT mice (gray squares) were infected intranasally with *Mp*. Control mice (white circles) received SP4 medium alone. (A) Body weight changes were monitored at indicated time points p.i. and expressed as percentage of body weight at day 0; the mean \pm SD of each group is shown ($n=6-13$ mice/time point). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid; values are expressed as median with IQR. (D) Histopathology score for mice at indicated time points p.i.; the mean \pm SD of each group ($n=6-7$ mice/time point) is shown. (E) Absolute number of leukocytes in

lungs at different days p.i. (F) Numbers of lung granulocytes (Ly6G⁺CD11b^{hi}CD11c⁻), monocytes (CD11b^{hi}Ly6G⁻CD11c⁻), CD4⁺ T cells (CD3⁺CD4⁺), and CD8⁺ T cells (CD3⁺CD8⁺). (G) IL-6, MCP-1, MIP1 β , and TNF- α in nasal lavage fluids of *Mp*-infected WT and μ MT mice were determined by multiplex fluorescent-bead-based immunoassay. Median levels with IQR are shown. * p <0.05, ** p <0.01 (A: Welch's t -test with Holm-Bonferroni method correction; B–C: Mann-Whitney U test with Holm-Bonferroni method correction; D–F: Kruskal-Wallis test with post hoc Dunn's multiple comparisons test; A–C: μ MT vs. infected WT mice).

Figure 6. Adoptive transfer of WT serum allows μ MT mice to control *Mp* infection in the lungs. (A) Experimental setup of the serum transfer experiment (n =6 mice/group). (B–C) Bacterial loads as CFU/ml in BALF and nasal lavage fluid of WT and μ MT mice. (D) *Mp*-specific IgG levels (AU/ml) in BALF of WT and μ MT mice. (E) Correlation between *Mp*-specific IgG and bacterial load in BALF of μ MT recipient mice that were passively immunized or control injected. The p value is indicated in the graphs (B–D: Mann-Whitney U test; E: Pearson product moment correlation coefficient).

Figure 1.

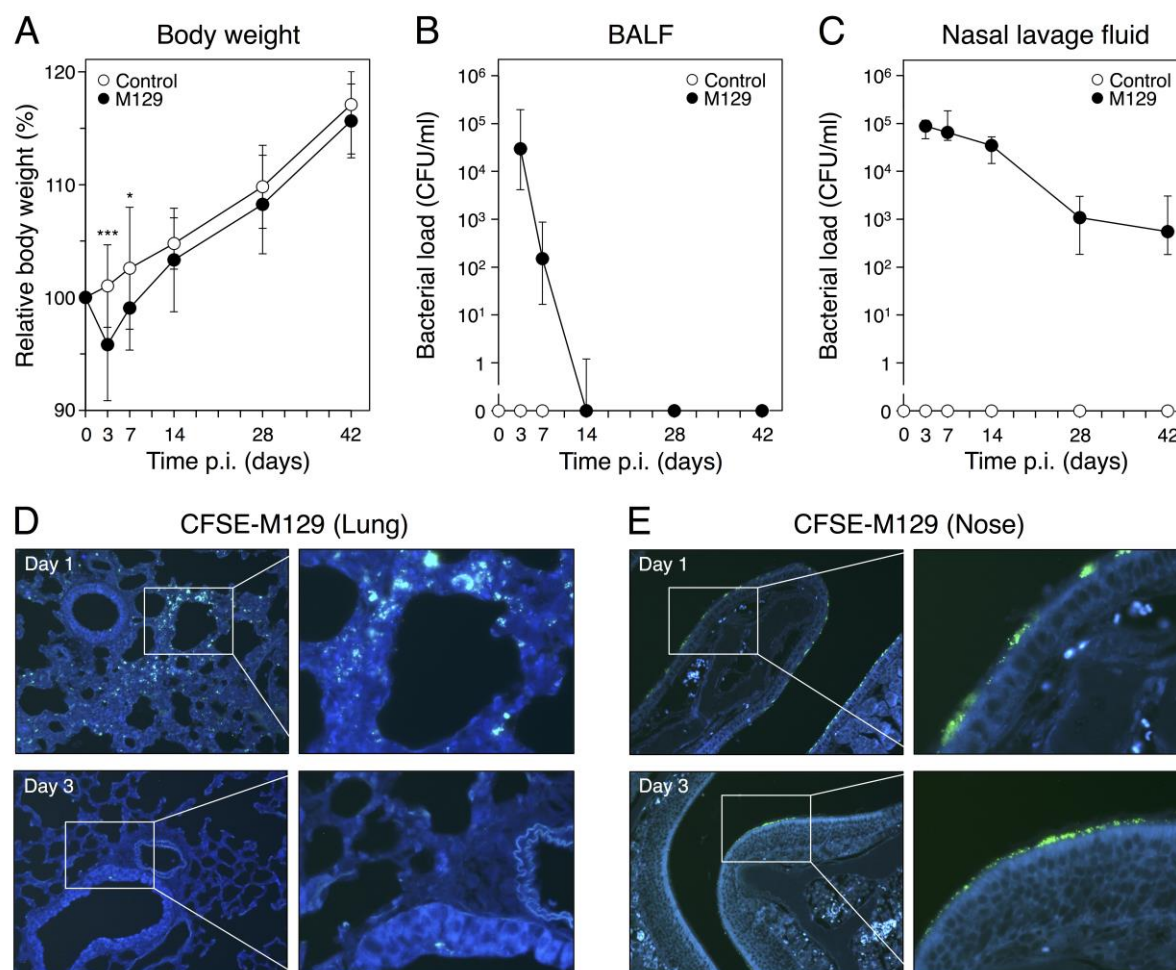


Figure 2.

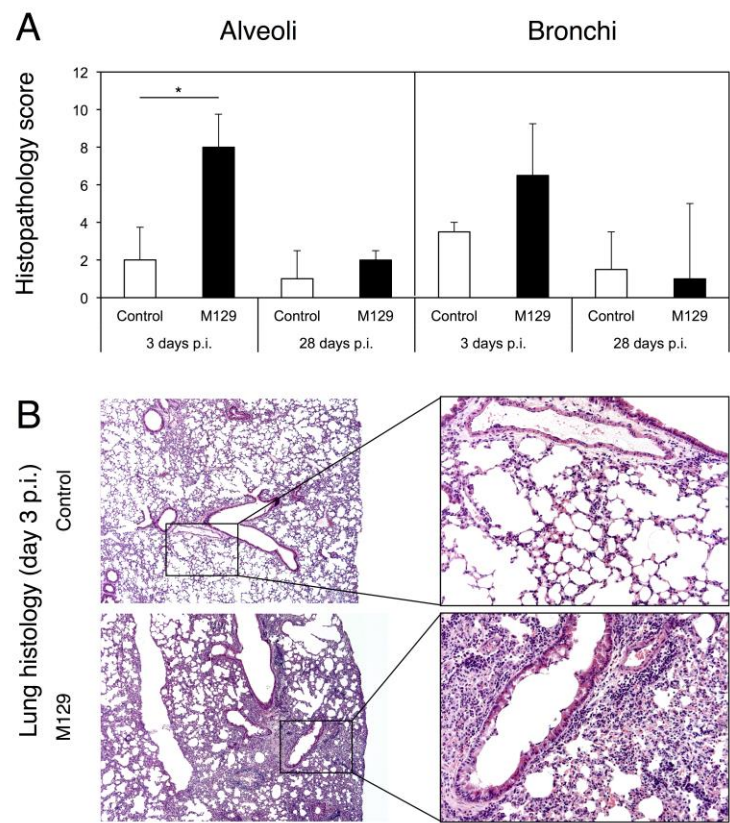


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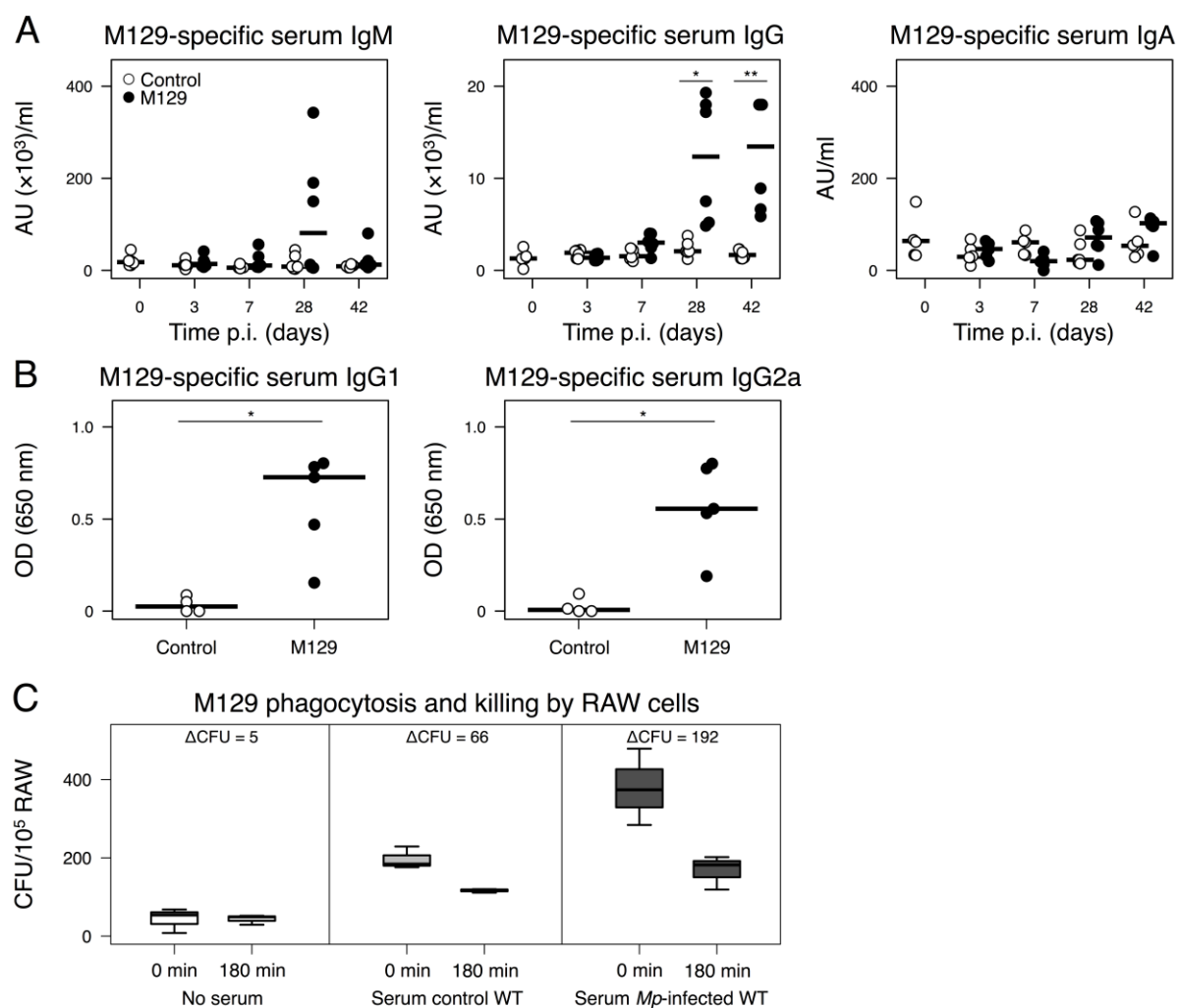


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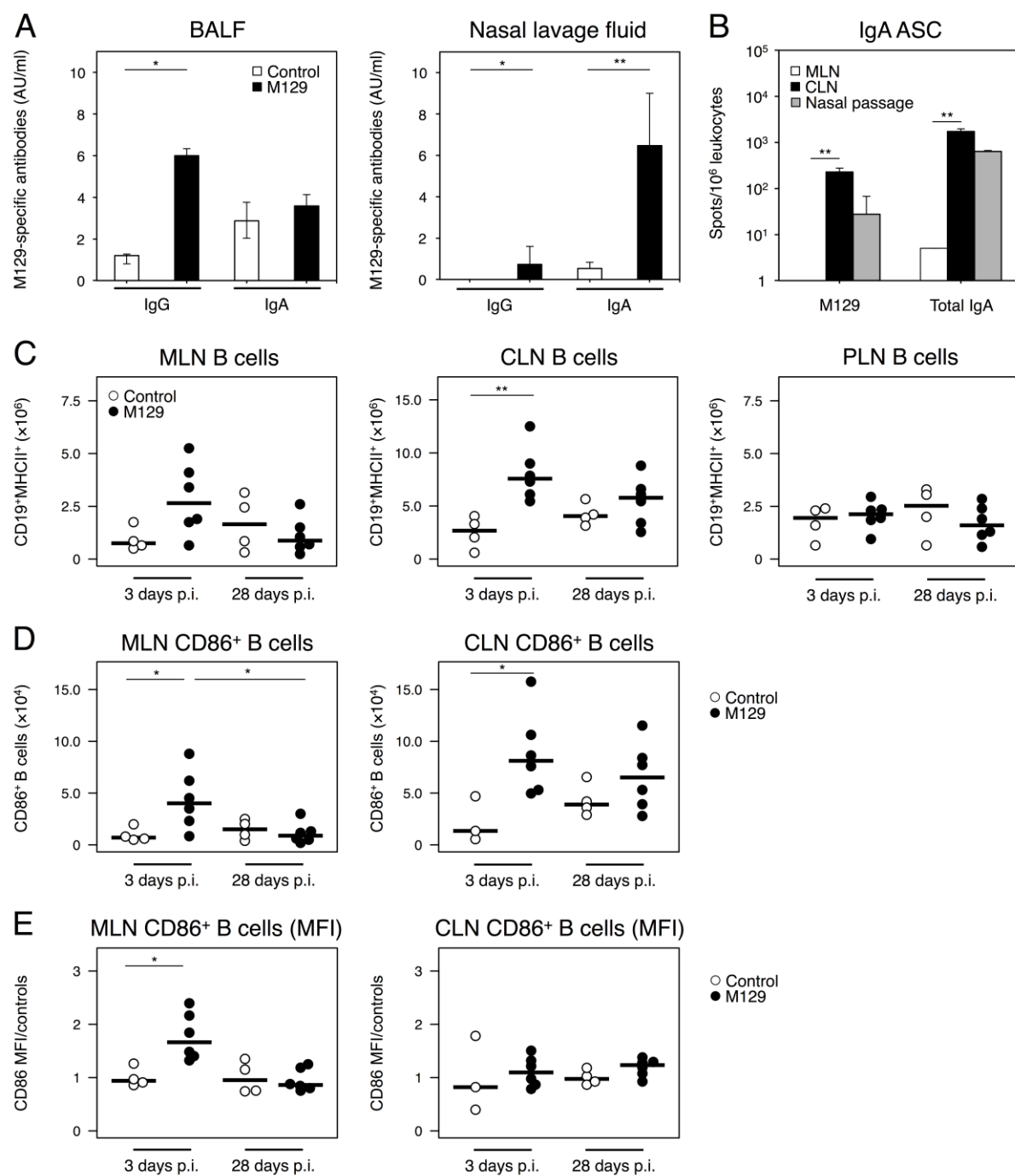


Figure 5.

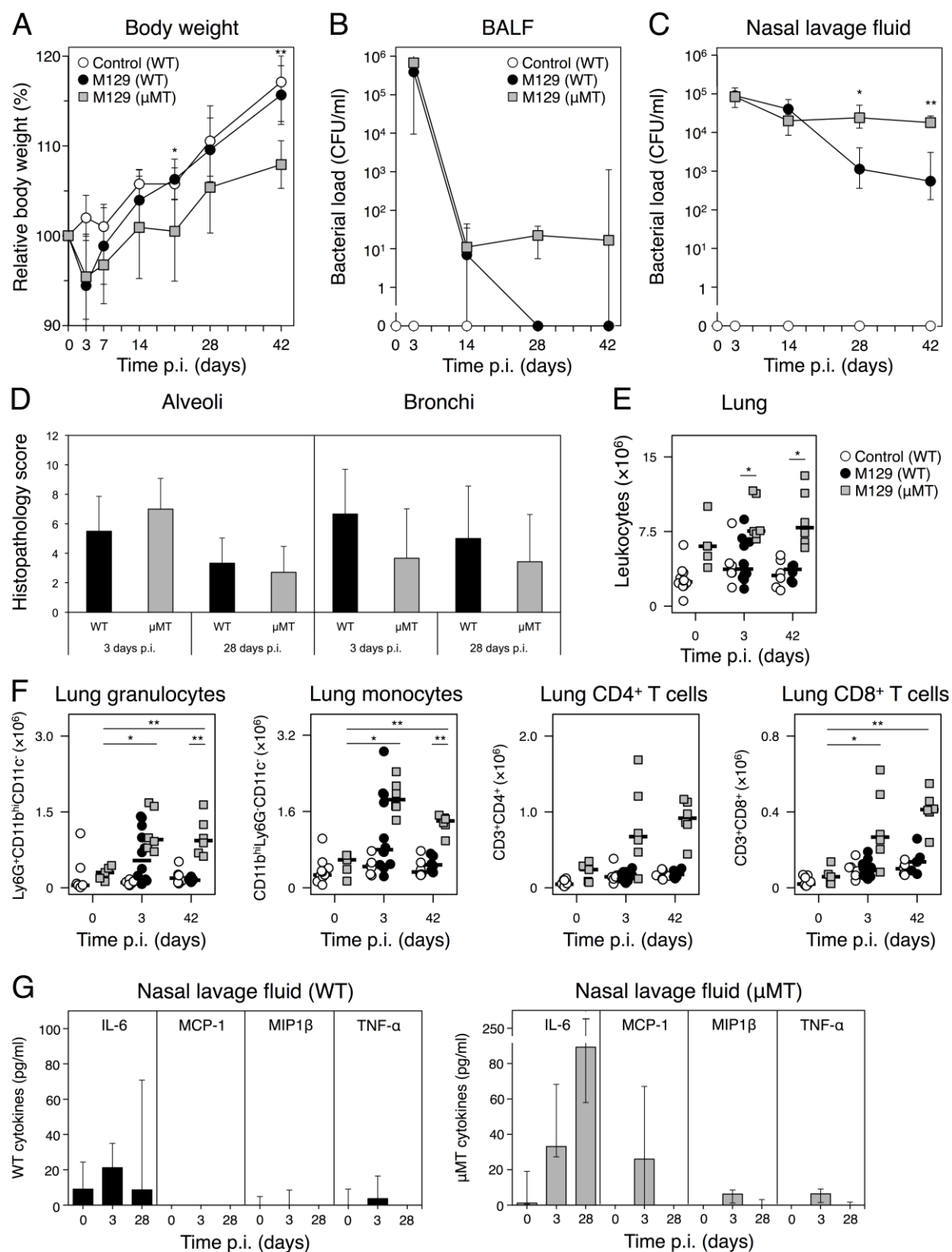


Figure 6.

